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The *Campylobacter jejuni* PhosS/PhosR operon represents a non-classical phosphate-sensitive two-component system

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Summary

The bacterial pathogen *Campylobacter jejuni* carries several putative two-component signal transduction systems of unknown function. Here we report that the PhosS (Cj0889) and PhosR (Cj0890) proteins constitute a two-component system that is activated by phosphate limitation. Microarray analysis, real-time RT-PCR, and primer extension experiments indicated that this system regulates 12 genes (including the *pstSCAB* genes) present in three transcriptional units. Gel shift assays confirmed that recombinant PhosR protein bound DNA fragments containing the promoter regions upstream of these three transcriptional units. Although functionally similar, the PhosS/PhosR does not exhibit sequence homology with the classical PhoBR systems, has a different *pho* box (5′-GTTTCNAAAANGTTTC-3′) recognized by the *C. jejuni* response regulator, and is not autoregulated. Because of these atypical properties, we designated the Cj0889-Cj0890 operon as the *C. jejuni* PhosS/PhosR system (phosphate sensor/phosphate response regulator) and the phosphate-regulated genes as the *pho* regulon of *C. jejuni*.

Introduction

Phosphorus (P) is an essential component for all living organisms. In bacteria, P typically is assimilated as inorganic orthophosphate (P_i) that is transported into the cell via specific uptake systems. Due to the scarcity of P_i in the natural world (Ozanne, 1980), organophosphates and phosphonates also may serve as P sources. These compounds are either degraded extracellularly where after the released P_i is taken up, or imported as complex molecules by specific uptake systems and degraded inside the cells.

Limitation of inorganic phosphate forces bacteria to synthesize a number of proteins involved in the assimilation of P_i from the environment. The genes encoding these proteins comprise the phosphate (*pho*) regulon that is controlled by the two-component signal transduction system PhoBR present in many bacterial species (named PhoP-PhoR in *Bacillus subtilis*) (Wanner, 1996; Hulett, 2002). In response to P_i limitation, the sensor PhoR autophosphorylates at a highly conserved histidine residue. The phosphoryl group is transferred to an aspartic acid residue in the N-terminal receiver domain of the response regulator PhoB (PhoP). This results in a conformational change that activates the C-terminal output domain, which then changes the cell physiology by modulating transcription. Phosphorylated PhoB or PhoP transcriptional factors activate the expression of more than 30 different genes in *Escherichia coli* and *B. subtilis*, respectively, by binding to the *pho* boxes located upstream of the phosphate-regulated genes (Martin, 2004). Some of these *E. coli* genes code for a high-ATP-driven P_i transport system (*pstSCAB*), a phosphatase (*phoA*), a negative PhoBR regulator protein (*phoU*), a polyanion transporter (*phoE*) and systems for the uptake and degradation of organophosphates (*ugpBAEC*) and phosphonates (*phnC-phnP*) (Wanner, 1996). Like in *E. coli,* the *B. subtilis* PhoPR system is autoregulated and activates the P_i transport system (*pstSCA* B_1 B_2) and phophatases (*phoA* and *phoB*). In response to P_i limitation, *B. subtilis* obtains phosphate by replacing its teichoic acid with teichuronic acid by activating the PhoPR-dependent genes *phoD* and *tuaABCDEFGH* and repressing the *tagABDEF* genes (Hulett, 2002).

Campylobacter jejuni is the leading causative agent of bacterial enteritis worldwide (Altekruse *et al*., 1999). It is widespread throughout nature and can be isolated from most warm blooded animals and from a wide variety of watery environmental sources (Rosef *et al*., 2001; Diergaardt *et al*., 2004). Analysis of the genome sequence of *C. jejuni* revealed the presence of 11 putative response regulators, six histidine sensor proteins and one

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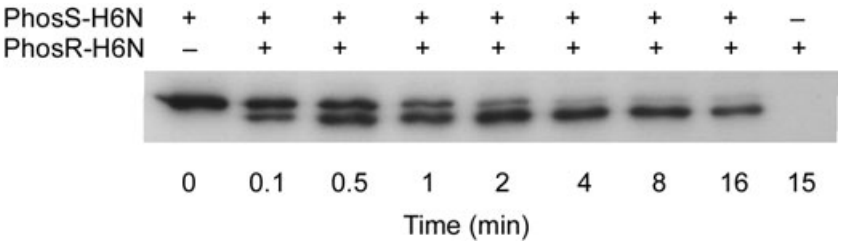

Fig. 1. Phosphorylation of the PhosR protein by the cytoplasmic domain of the PhosS protein *in vitro*. Autophosphorylation of the truncated sensor PhosSc-H6N (22 pmol) was accomplished by incubation of PhosSc-H6N with [γ-^{32}P]ATP for 15 min at room temperature. Subsequently, 4.3 pmol PhosR-H6N was added to the phosphorylated PhosSc-H6N protein in a final volume of 80 µl. At the indicated time points, 10 µl samples were taken and the phosphate transfer reaction was stopped by adding SDS loading buffer. The PhosR-H6N protein was also incubated for 16 min with [γ-^{32}P]ATP without the sensor PhosSc-H6N. The samples were run on a 12.5% SDS-polyacrylamide gel, and autophosphorylation and phosphotransfer were visualized by autoradiography.

hybrid sensor response regulator protein (Parkhill *et al*., 2000). Five of the genes encoding *C. jejuni* response regulators, *cheY*, *flgR*, *racR*, *dccR* and *cbrR,* have been characterized to date (Yao *et al*., 1997; Bras *et al*., 1999; MacKichan *et al*., 2004; Wösten *et al*., 2004; Raphael *et al*., 2005). Based on amino acid sequence neither a PhoBR homologue is present, nor genes known to be regulated by the *E. coli* PhoBR or *B. subtilis* PhoPR two-component systems, except for the high-ATP-driven P_i transport system (*pstSCAB*). This lack of homology suggests that the *C. jejuni pstSCAB* genes may be regulated by a different mechanism.

In the present study, we provide evidence that the *C. jejuni* PhosS (Cj0889) and PhosR (Cj0890) proteins constitute a two-component system that, in response to P_i limitation activates (in a direct fashion) the *pstSCAB* genes and eight other genes. The PhosS/PhosR system shows no sequence homology with and lacks typical features of other phosphate-sensitive two-component systems. Our results suggest that the *C. jejuni* system may have evolved independently from thus far identified phosphate-sensitive systems in other prokaryotes.

Results

The PhosS and PhosR proteins represent a two-component system

The *Campylobacter* PhosS and PhosR proteins show structural similarities to the family of two-component sensor and response regulator proteins respectively (Parkhill *et al*., 2000). To confirm that PhosS and PhosR constitute a two-component system we performed phosphorylation assays. Hereto the PhosS and PhosR proteins were expressed in *E. coli* and isolated as His-tagged recombinant proteins. Phosphorylation assays with the purified proteins demonstrated that the cytoplasmic domain of the sensor PhosS was able to rapidly autophosphorylate itself in the presence of radioactive ATP (Fig. 1). Addition of recombinant regulator PhosR resulted in a rapid dephosphorylation of the sensor. After 4 min of incubation most of the phosphate was transferred from the sensor to the regulator. The total amount of labelled phosphate associated with the sensor and regulator decreased during the course of the experiment, indicating that one of the proteins might have phosphatase activity. This phosphate transfer shows that PhosS and PhosR communicate with each other and represent a two-component system.

The gene pstS *(Cj0613) is regulated by the two-component system PhosS/PhosR*

To address the role of the PhosS/PhosR two-component system in *C. jejuni,* we made a mutation in the response regulator PhosR, by insertion of a chloramphenicol resistance (Cm^r) cassette. To identify the genes regulated by the PhosS/PhosR system, RNA of logarithmic grown [Heart Infusion (HI) broth] wild-type and *phosR*::Cm mutant bacteria was isolated and RNA levels were compared using microarray analysis. The microarrays used cover 94% of the predicted open reading frames (ORFs) of the *C. jejuni* strain 11168 and an additional 276 *C. jejuni* genes not present in the *C. jejuni* 11168 genome. Thirteen genes were more than twofold up- or downregulated in the *phosR*::Cm mutant compared with the parent strain (data not shown). The differences in mRNA levels for these genes were also examined by real-time RT-PCR, normalized to the level of *gyrA* transcript. Most of the genes identified by the microarray analysis (like Cj1184) could not be confirmed by real-time RT-PCR; however, the *pstS* (Cj0613) gene was clearly downregulated in the *phosR*::Cm mutant (Fig. 2A).

Phosphate limitation promotes the transcription of the pstS *gene*

Transcription of *pstS* in other bacteria is inducible by phosphate limitation (Allenby *et al*., 2004). To investigate whether this is true for the *C. jejuni pstS* gene, we performed real-time RT-PCR on RNA isolated from wild-type and *phosR*::Cm mutant bacteria grown in low phosphate-containing medium. Transcription of the *pstS* gene in the

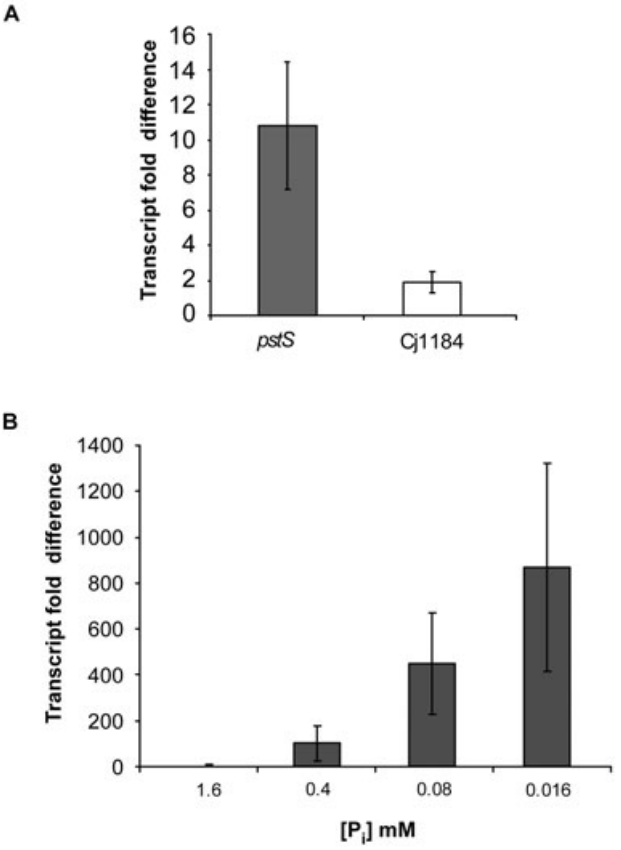

Fig. 2. Real-time RT-PCR analysis showing that transcription of the *pstS* gene is PhosS and [P_i] dependent.
A. Expression analysis by real-time RT-PCR of genes predicted by microarray to be regulated by the response regulator PhosR. RNA used in the real-time RT-PCR was isolated from wild type and *phosR*::Cm mutant grown in HI broth at logarithmic phase at 37°C. Data shown are fold changes in transcript (*phosR*::Cm mutant strain relative to wild type) normalized to *gyrA* transcript for the selected genes.
B. Transcripts of the *pstS* gene in the wild type and *phosR*::Cm mutant grown in defined media containing 1.6, 0.4, 0.08 or 0.016 mM [P_i] were measured by real-time RT-PCR. Data shown are the *pstS* transcript of the *phosR*::Cm mutant strain relative to wild type normalized to *gyrA* transcript. Data represent the mean values and standard deviation of four independent experiments with at least two independent preparations of RNA.

wild type and *phosR*::Cm mutant was similar when bacteria were grown in defined medium containing 1.6 mM [P_i] (Fig. 2B). In contrast, the *pstS* transcription levels differed more than 800-fold when the wild-type and mutant strains were grown in defined media with 0.016 mM [P_i] due to upregulation of *pstS* transcription in the wild-type strain but not in the mutant.

The PhosS/PhosR system is required for optimal growth in phosphate-limited media

To examine whether phosphate limitation influenced the growth kinetics of the *phosR*::Cm mutant compared with the wild-type strain, we measured the optical densities of the wild-type and mutant bacteria grown under reduced phosphate concentrations. Phosphate limitation clearly resulted in a lower optical density for all strains, indicating that [P_i] was the limiting factor in the media (Fig. 3). Growth curves of the strains were similar in defined media containing 1.6 mM [P_i]. In media containing 0.08 or 0.016 mM [P_i], the initial growth rate of the strains was also the same; however, the *phosR*::Cm mutant reached a final lower optical density faster. To verify that the disruption of the *phosR* gene was responsible for the lower final optical density, we complemented the *phosR*::Cm strain. Hereto plasmid pCP890, containing the complete sensor *phosS* and response regulator *phosR* gene, was introduced into the *phosR*::Cm mutant. The growth curve of the complemented strain was similar to that of the wild type in all tested media, indicating that the mutation of PhosR is responsible for the lower final optical density under phosphate-limiting conditions.

Twelve genes are regulated by the PhosS/PhosR system

To identify the complete regulon belonging to the PhosS/PhosR system, we repeated the microarray analysis, but this time using RNA isolated under PhosS/PhosR inducing conditions (0.08 mM [P_i]). Twenty-five genes were more than 2.5-fold up- or downregulated in the wild type compared with the *phosR*::Cm mutant. Of these 25 genes, five genes, Cj0145, *pstS*, *pstC*, Cj0728 and Cj0729, were more than 70-fold upregulated (Fig. 4A). Genome analysis showed that the *pstS* and Cj0728 genes were the first of a series of three and five downstream genes respectively, that were also shown to be dependent on a functional PhosS/PhosR system. Most likely, the genes *pstSCAB* (Cj0613-Cj0616) and Cj0728-Cj0733 form two PhosS/PhosR-regulated operons. All transcripts that were more than 4.5-fold up- or downregulated by microarray were verified by real-time RT-PCR. As a result, three operons Cj0145, *pstSCAB* and Cj0728-Cj0733 appeared to be regulated by PhosS/PhosR system (Fig. 4B). Based on the gene organization however, we expected Cj0727 rather than Cj0728 to be the first gene of the Cj0727-Cj0733 operon. Unfortunately, Cj0727 was missing from the microarray. Therefore, we also performed real-time RT-PCR on this gene. Transcription of the Cj0727 gene was strongly reduced in the *phosR*::Cm mutant as was observed for the Cj0728 gene. From these results we conclude that 12 genes, Cj0145, *pstSCAB* and Cj0727-Cj0733, located in three putative operons, depend on a functional PhosS/PhosR system, forming the *pho* regulon of *C. jejuni*.

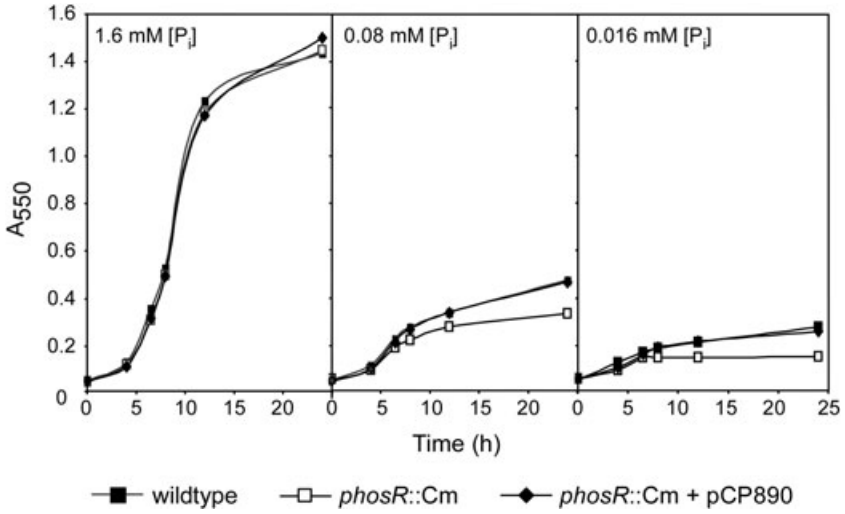

Fig. 3. Growth curve demonstrating that a functional PhosS/PhosR system is required for optimal growth during phosphate starvation. Optical densities (OD_{550}) of the wild type, *phosR*::Cm mutant or *phosR*::Cm mutant containing complementation plasmid pCP890, were determined at different times following inoculation in defined medium containing 1.6, 0.08 or 0.016 mM [P_i]. The graph shown is representative of three independent experiments.

PhosS/PhosR regulates an alkaline phosphatase

To confirm that the observed regulation of transcription can be extrapolated to the protein level, one of the regulated genes was studied in more detail. In *E. coli* and other bacteria, limitation of [P_i] induces a PhoB/PhoR-dependent alkaline phosphatase PhoA (Wanner, 1996; Hulett, 2002). Although a homologue of the *E. coli* PhoA appears to be absent in the *C. jejuni* genome, we investigated whether the PhosS/PhosR system is regulating an unknown alkaline phosphatase. To examine this, the wild type and the *phosR*::Cm mutant were grown in defined medium with 1.6, 0.4, 0.08 or 0.016 mM [P_i] and subjected to an alkaline phosphatase assay. A clear increase in phosphatase activity was observed for the wild-type bacteria as the phosphate concentration decreased (Fig. 5). No phosphatase activity was measured for the *phosR*::Cm mutant, indicating that the PhosS/PhosR is regulating a phosphatase. Of the 12 genes regulated by the PhosS/PhosR system, gene Cj0145, annotated as hypothetical protein in the database, showed some similarity with the alkaline phosphatase of *Vibrio cholerae*. To determine if the gene Cj0145 is coding for the phosphatase, we inactivated this gene. Alkaline phosphatase assays of the Cj0145 mutant revealed that this gene is responsible for the alkaline phosphatase activity in the wild type (Fig. 5). These phosphatase assays confirm the mRNA induction seen for the PhosS/PhosR-regulated genes in response to phosphate starvation.

Complementation in trans *of the* phosR*::Cm mutant results in a wild-type mRNA profile*

To verify that disruption of *phosR* was responsible for the transcription defect of the above-mentioned 12 genes, we complemented the *phosR*::Cm strain. Hereto plasmid pCP890, containing the complete sensor *phosS* and response regulator *phosR* genes, was introduced into the *phosR*::Cm mutant. Transcripts of the Cj0145, *pstS* and Cj0727 genes in the wild type, mutant and complemented mutant grown under phosphate rich (1.6 mM) or poor (0.08 mM) conditions were determined by real-time RT-PCR (Fig. 6). Only minor transcript fold differences were observed between wild type and mutant or the complemented strain in phosphate-rich defined medium. However, transcript differences of more than 200-fold between the wild-type and mutant strains for the Cj0145, *pstS* and Cj0727 genes were seen at low [P_i] while less than 30-fold in the case of the wild-type and complemented strain. Taken together, these results demonstrate that plasmid pCP890 is able to complement the defect in the *phosR* gene and thus that PhosR is involved in the regulation of the *C. jejuni pho* regulon.

Mapping of the transcriptional start sites of promoters located upstream Cj0145, pstS *and Cj0727 genes*

To identify the PhosR-dependent transcription start sites in the Cj0145, *pstS* and Cj0727 promoter regions, we performed primer extension experiments using total RNA isolated from the wild-type and the *phosR*::Cm mutant strains grown under phosphate starvation conditions. Primer extension products were obtained only when RNA isolated from the wild-type cells was used (Fig. 7A), consistent with the real-time RT-PCR data for these genes. Two adjacent PhosR-dependent transcription start points were obtained for the Cj0145 promoter corresponding to a T or G residue 30 or 29 bp upstream of the Cj0145 translational start site (Fig. 7A and F). Single PhosR-dependent transcription start sites were identified for the *pstS* and Cj0727 promoters, corresponding to a C residue 23 bp upstream of the *pstS* translation start codon and to

A

Genes upregulated in the wildtype compared to *phosR*::Cm mutant

Gene designation	Putative function	Change in expression level (n-fold)
Cj0145	putative phosphatase	71.4
Cj0613	Phosphate ABC transporter, periplasmic phosphate-binding protein,PstS	106.2
Cj0614	Phosphate ABC transporter, permease protein,PstC	90.6
Cj0615	Phosphate ABC transporter, permease protein, PstA	12.7
Cj0616	Phosphate ABC transporter, ATP-binding protein, PstB	10.8
Cj0728	putative periplasmic protein	79.7
Cj0729	Conserved hypothetical protein	84.5
Cj0730	possible ABC transporter (permease protein)	41.5
Cj0731	possible ABC transporter (permease protein)	23.5
Cj0732	possible ABC transporter (ATP-binding protein)	14.9
Cj0733	putative HAD-superfamily hydrolase	5.3
Cj1148	ADP-heptose--LPS heptosyltransferase II, WaaF	4.5

Genes downregulated in the wildtype compared to *phosR*::Cm mutant

Gene designation	Putative function	Change in expression level (n-fold)
Cj0429c	Conserved hypothetical protein	7.9
Cj1012c	Conserved hypothetical membrane protein	5.2

B

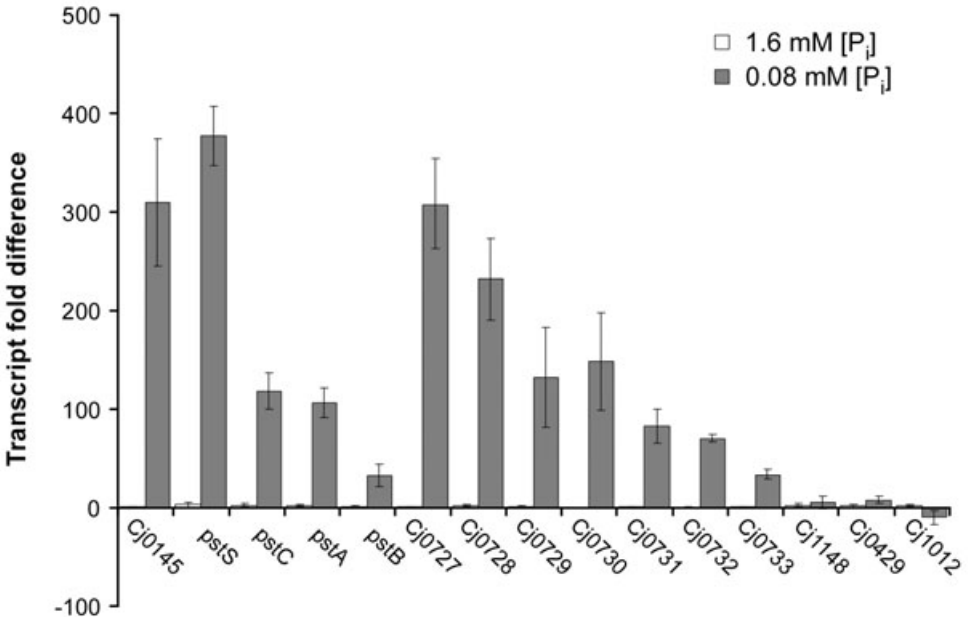

Fig. 4. Gene expression profiling of total RNA of *phosR*::Cm and wild type grown under phosphate-limiting conditions.
A. Microarray data obtained by comparison of total RNA isolated of wild type and *phosR*::Cm mutant grown under phosphate-limiting (0.08 mM) conditions. Fold transcript differences of genes measured in the *phosR*::Cm mutant strain relative to wild type are shown.
B. Verification of differentially expressed genes as identified by low [P_i] microarray by real-time RT-PCR. Shown are fold transcript differences of the indicated genes in the *phosR*::Cm mutant strain relative to wild type normalized to *gyrA* transcript. Data represent the mean values and standard deviation of four independent experiments with two independent preparations of RNA.

an A residue 21 bp upstream of the Cj0727 translation start codon. Upstream the transcription start sites of these genes, a −10 promoter region is located displaying similarity to the *C. jejuni* σ^{70} −10 promoter region (Wösten *et al*., 1998); however, no conserved σ^{70} −35 promoter region could be identified (Fig. 7F).

Identification of the C. jejuni pho *box located in front of the Cj0145,* pstS *and Cj0727 genes by gel mobility shift assays*

To investigate whether PhosR is able to bind to the promoter regions upstream the Cj0145, *pstS* and Cj0727

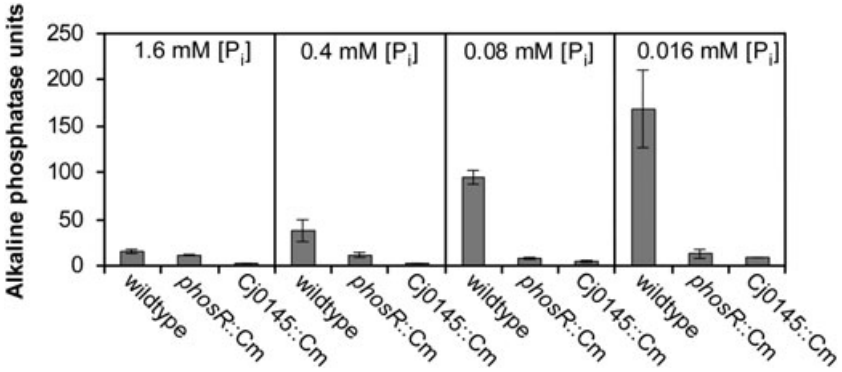

Fig. 5. Alkaline phosphatase activity in wild-type, *phosR*::Cm and Cj0145::Cm mutant strains. Alkaline phosphatase activity was measured in wild-type, *phosR*::Cm or Cj0145::Cm cells grown in defined medium containing 1.6, 0.4, 0.08 or 0.016 mM [P_i]. Data correspond to mean values of two independent experiments done in duplicate. Error bars correspond to the standard deviations.

genes, we performed gel mobility shift assays with PhosR. The PhosR response regulator protein was isolated as his-tagged recombinant protein (PhosR-His) and incubated (in non-phosphorylated form) with DNA fragments containing the promoter regions upstream of *pstS*, Cj0145 or Cj0727. The PhosR-His protein was able to shift the DNA fragments pstS-F1 and pstS-F2 containing the *pstS* promoter with or without the −10 region respectively (Fig. 7B and C). The formation of the PhosR-His protein and pstS-F1 DNA complex was efficiently inhibited in the presence of an excess of unlabelled pstS-F1 fragment, indicating specificity of the interaction (Fig. 7D). No band shifts were detected when a DNA fragment pstS-F3 lacking the −10 and −35 regions of the *pstS* promoter was used (Fig. 7C). The difference between the DNA fragments pstS-F2 and pstS-F3 is only 33 bp comprising the putative −35 promoter region of the *pstS* gene. Based on the gel shift and primer extension experiments, the results suggest that the PhosR-His recombinant protein binds to the DNA sequence overlapping the putative −35 region. The promoter regions of Cj0145 and Cj0727 could not be shifted by PhosR-His recombinant protein alone, indicating that the PhosR-His recombinant protein has a higher affinity for the *pstS* promoter region than for the Cj0145 and Cj0727 promoter regions (Fig. 7E). The recombinant sensor PhosS-His protein together with ATP is needed to observe a band shift for the Cj0145 and Cj0727 promoter regions (Fig. 7E). Alignment of the three promoter regions showed a strong conserved region around the −35 putative promoter regions resembling the sequence GTTTC NAAAANGTTTC (Fig. 7F). This sequence is located in the 33 bp extra DNA present in pstS-F2 compared with pstS-F3 which we designated as the *pho* box of *C. jejuni*.

Discussion

Bacteria monitor changes in their environment to be optimally adapted to their ecological niches. The sensing and responding to external stimuli is usually mediated by two-component signal transduction systems. Here we provide evidence that (i) the bacterial pathogen *C. jejuni* PhosS and PhosR proteins form a two-component system, (ii)

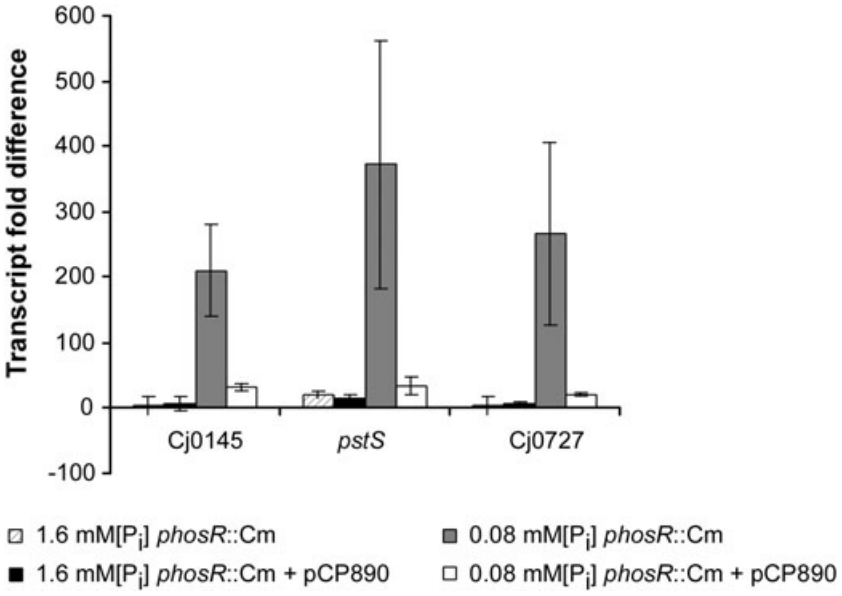

Fig. 6. Complementation of the *phosR*::Cm mutant as shown by real-time RT-PCR analysis. Quantitative real-time RT-PCR was used to measure the transcripts of the Cj0145, *pstS* and Cj0727 genes in wild type, *phosR*::Cm mutant or *phosR*::Cm mutant containing complementation plasmid pCP890. Strains were grown in defined medium containing 1.6 or 0.08 mM [P_i]. Data shown are transcript difference of indicated genes in the mutant or complemented mutant relative to the wild type normalized for the *gyrA* transcript. Data represent the mean values and standard deviation of four independent experiments with two independent preparations of RNA.

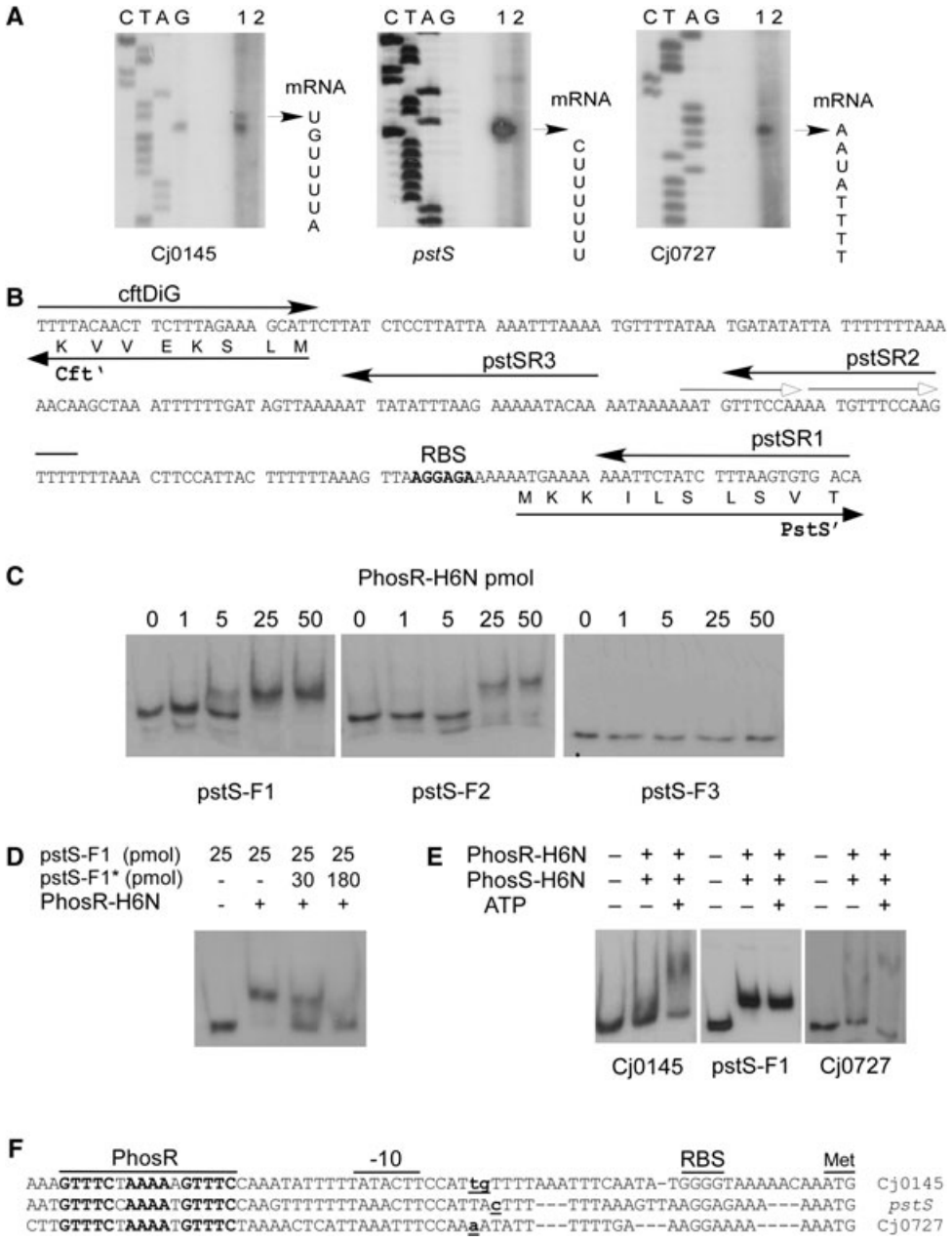

Fig. 7. Characterization of the promoter regions upstream the Cj0145, *pstS* and Cj0727 genes.
A. Primer extension experiments were performed to determine the transcription start sites upstream of the Cj0145, *pstS* and Cj0727 genes. Primer extension products were generated using total RNA isolated from wild type (lane 1) or *phosR*::Cm mutant (lane 2) bacteria grown in defined medium containing 0.08 mM [P_i]. The primer extension products were run on a 6% sequencing gel against dideoxy sequencing reactions primed with the same primer as used for the extension reactions. The sequence spanning the transcription start site is shown, and the transcription start site is marked with an arrow.
B. DNA sequence of the intermediate region between the genes *cft* and *pstS*. Coding sequence, ribosomal binding sequence (RBS) and primers used for gel mobility shift assays are indicated.
C. Gel mobility shift assays of *pstS* promoter fragments bound by PhosR-H6N protein. Dig-labelled PCR fragments pstS-F1 (232 bp), pstS-F2 (163 bp) and pstS-F3 (130 bp) made with primers sets CFTDig/pstSR1, CFTDig/pstSR2 and CFTDig/pstSR3 respectively, and incubated with 0, 1, 5, 25 or 50 pmol of PhosR-H6N protein. The concentration of PhosR-H6N protein used in the reactions is indicated above the lanes.
D. Competition gel mobility shift assays were performed by incubation of 25 pmol PhosR-H6N protein with 25 pmol labelled pstS-F1 and different indicated concentrations of unlabelled pstS-F1* DNA fragments.
E. Gel mobility shift assays of the Cj0145 *pstS* and Cj0727 promoter regions bound by PhosR-H6N. Dig-labelled PCR fragments containing the Cj0145, *pstS* and Cj717 promoter region were incubated with 25 pmol PhosR-H6N and 44 pmol PhosS-H6N with or without 2 mM ATP.
F. Alignment of the Cj0145, *pstS* and Cj0727 promoter DNA sequences. Start codon (Met), ribosomal binding sequence (RBS) experimental determined transcription startpoints (small capital) and putative −10 regions (−10) as well as the putative PhosR binding sequence (PhosR) are shown.

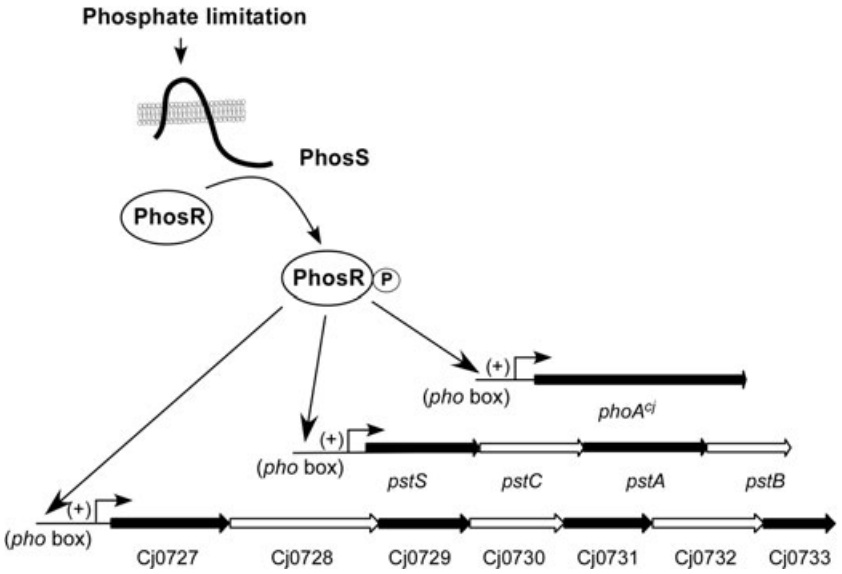

Fig. 8. Model illustrating the three transcriptional units directly regulated by the *C. jejuni* PhosS/PhosR two-component system. The PhosR protein promotes the transcription of the 12 indicated genes during growth of *C. jejuni* in phosphate-limited media.

this system is activated during phosphate starvation, (iii) this system is required for optimal growth under phosphate limitation, (iv) this system regulates 12 genes involved in the acquisition of P_i, (v) (GTTTC NAAAANGTTTC) is likely to be the PhosR binding sequence and (vi) the Cj0145 gene of unknown function is coding for an alkaline phosphatase (Fig. 8).

Evidence that the PhosS and PhosR genes comprise a two-component system was provided by the autophosphorylation and phosphate transfer assays with purified PhosS and PhosR recombinant proteins (Fig. 1). The rapid phosphorylation of PhosS in the presence of ATP indicated that the protein carried autokinase activity, which is typical for two-component sensor kinase proteins. In many other two-component systems, phosphorylated response regulators are less stable due to phosphatase activity of the regulator or sensor protein (Stock *et al*., 2000; Wösten *et al*., 2004). Our results suggest that the PhosS/PhosR two-component system also may possess phosphatase activity. Because both the sensor PhosS and response regulator PhosR do not show any obvious sequence similarities with other known members of the family of two-component proteins (Stock *et al*., 2000), we decided to perform microarray analysis to identify the regulon that is activated by this system. The identification of the gene annotated as *pstS* and supposedly coding for a protein involved in phosphate uptake led us to investigate whether low phosphate levels could activate this system. We were able to demonstrate that the PhosS-PhosR system was activated and required for bacterial growth during phosphate starvation and that its induction resulted in the expression of 12 genes, representing the *pho* regulon of *C. jejuni*. In many other bacterial species, genes involved in the assimilation of P_i from various phosphorus compounds are under the control of the PhoBR two-component signal transduction system (Wanner, 1996; Hulett, 2002). Although functionally similar (i.e. regulated by phosphate), the PhosS/PhosR and classical PhoRB systems exhibit major differences: (i) in contrast to PhoR, the sensor PhosS contains a large periplasmic domain but no PAS domain (Koretke *et al*., 2003), (ii) the DNA motif (*pho* box) recognized by the *C. jejuni* response regulator does not resemble that of PhoB, although they both do recognize a direct repeat, (iii) PhosR-regulated promoters contain only one *pho* box, (iv) the PhosS/PhosR system is not autoregulated as no difference in the amount of PhosS transcript were observed between the wild type and *phosR*::Cm mutant grown at low [P_i] (data not shown) and (v) in addition, the negative PhoBR regulator PhoU appears absent in *C. jejuni* as deduced from BLAST analysis and the microarray results. Based on these differences we proposed to rename Cj0889 as PhosS (phosphate sensor) and Cj0890 as PhosR (phosphate response regulator) instead of using the symbols PhoB/PhoR.

We identified three transcriptional units that are regulated by the PhosS/PhosR system by microarray analysis, real-time RT-PCR analysis, and primer extension. The response regulator PhosR binds to the sequence overlapping the −35 promoter regions upstream of these units. The DNA shift experiments suggest that the protein has a higher affinity for the *pstS* than for the Cj0145 and Cj0727 promoter regions (Fig. 7C). It is known that phosphorylation of response regulators increases the binding affinity to their target promoters (Cho *et al*., 2001). While the three promoter regions share a common small direct repeat (GTTTC) separated by a conserved polyA region,

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
C. jejuni strains		
81116	Wild type	Palmer *et al.* (1983)
phosR::Cm	81116 derivative *phosR*::Cm	This study
Cj0145::Cm	81116 derivative Cj0145::Cm	This study
E. coli strains		
PC2955	relA1 Φ80dlacZΔM15 phoA8 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS glnV44	NCCB
BL21(DE3)	ompThsdS dcm$^+$ Tet^r gal(DE3) endA het	Novagen
Plasmids		
PGEM-T Easy	PCR cloning vector, Amp^r	Promega Corporation, Madison, WI
pAV35	pBluescript II SK containing *C. coli* Cm^r cassette	van Vliet *et al.* (1998)
pGEM890-889	pGEM-T Easy containing the *phosR* and *phosS* genes on a 3580 bp fragment	This study
pGEM890::Cm	pGEM890 with Cm^r inserted in *phosR*	This study
pGEM145	pGEM-T Easy containing gene Cj0145 on a 3230 bp fragment	This study
pGEM145::Cm	pGEM145 with Cm^r inserted in Cj0145	This study
pT7.7	Expression vector, Amp^r	Tabor and Richardson (1985)
pT7-889c-H6N	pT7.7 containing the truncated *phosS* gene with N-terminal His-taq	This study
pT7-890-H6N	pT7.7 containing *phosR* gene with N-terminal His-taq	This study
pGEM1491	pGEM-T Easy containing the Cj1493, Cj1492 and Cj1491 genes on a 2549 bp fragment	This study
pWM1007	*C. jejuni/E. coli* shuttle vector	Miller *et al.* (2000)
pWM1007Pr1492	pWM1007 containing the Cj1492 promoter	This study
pCP890	*phosS* and *phosR* genes downstream the 1492 promoter	This study

the direct repeat in the *pstS* promoter sequence is much larger: AATGTTTCCAA, which might explain the affinity difference for the target promoter elements. Scanning the *C. jejuni* genome for PhosR-binding sequence (GTTTCNAAAANGTTTC) did not lead to the discovery of additional PhosR-dependent promoter elements. Based on the microarray results and genome search for PhosR-binding sequences, we assume that the identified phosphate-sensitive genes constitute the complete *C. jejuni pho* regulon.

Further analysis of the genes comprising the *C. jejuni pho* regulon revealed that the gene Cj0145 is coding for an alkaline phosphatase (Fig. 5). This protein is 52% identical at the amino acid level to the *V. cholerae* PhoA^{VC}. This protein is a monomeric alkaline phosphatase that is different from the well-studied PhoA of *E. coli* (Majumdar *et al.*, 2005). To avoid confusing symbols we have named gene Cj0145, *phoA^{CJ}.* The four genes Cj0613-Cj0616 code for the high-ATP-driven P_i transport system PstSCAB (Wanner, 1996) that can be found in many bacterial species (Yuan *et al.*, 2006). Although the predicted amino acid sequences of the remaining seven genes in the *pho* regulon (Cj0727-Cj0733) are all highly conserved among other bacterial species, their function still needs to be determined (Fig. 4A) (Parkhill *et al.*, 2000). Four of these genes, however, appear to encode ABC transporters that might be part of an unknown uptake system.

Where does *C. jejuni* encounter low phosphate conditions? The natural habitat of *C. jejuni* is the intestine of warm-blooded animals and a wide variety of watery environmental sources. We have tested the colonization behaviour of the *phosR* mutant in the most favourite habitat of *C. jejuni,* the chicken gut. Colonization of this mutant was similar to that of the wild type (data not shown). Microarray analysis of RNA isolated from wild type and *phosR* mutant present in the chicken caeca revealed only a threefold difference of the *pstS* gene, while no differences were observed for the other PhosS/PhosR-regulated genes (data not shown). These data confirm the *in vivo* microarray data performed in chickens and rabbits in which none of the PhosS/PhosR-regulated genes were upregulated (Stintzi *et al.*, 2005; Woodall *et al.*, 2005). Phosphate concentrations of river water has been estimated to contain an average P_i of 0.18 mM (Hem, 1985). Therefore, we assume that the PhosS/PhosR system is needed to survive the less favoured *Campylobacter* habitat, surface water.

Experimental procedures

Strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *C. jejuni* 81116 and derivatives were routinely maintained at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) either on blood agar base II medium (Oxoid, London, UK) containing 5% horse blood lysed with 0.5% saponin (Sigma, St Louis, MO) or in HI broth (Oxoid). *E. coli* were grown in Luria–Bertani medium at 37°C. When appropriate, growth media were supplemented with ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}) or kanamycin (50 μg ml^{-1}).

Construction of the phosR *mutant*

A 3580 bp DNA fragment, containing the complete *phosR* and *phosS* genes, was amplified from the *C. jejuni* 81116 chromosome using the primers Cj0890cF and Cj0890cR (Table 2). This fragment was cloned into pGEM-T Easy (Promega Corporation, Madison, WI) resulting in plasmid pGEM890-889. Plasmid pGEM890-889 was subsequently amplified with primers Cj0890FBamHI and Cj0890RBamHI to introduce a BamHI restriction site. The PCR product was digested with BamHI and ligated to a 0.7 kb BamHI fragment containing a Cm^r gene of pAV35, resulting in the knockout construct pGEM890::Cm. This knockout construct containing the *phosR* gene with a 10 bp deletion and the Cm^r gene in the same orientation as the *phosR* gene was introduced by natural transformation in *C. jejuni* 81116 (Wassenaar *et al*., 1993). Homologous recombination resulting in double-crossover event was verified by PCR.

Construction of the Cj0145 mutant

The Cj0145 gene with flanking regions was amplified from the 81116 genome by PCR using the primers Cj144F2 and trxB (Table 2). The resulting 3230 bp fragment was cloned into pGEM-T Easy to form plasmid pGEM145. This plasmid was digested with XbaI and ClaI (resulting in a Cj0145 deletion of 174 bp) and ligated to a 0.7 kb XbaI-AccI fragment containing a Cm^r gene of pAV35. The resulting knockout construct pGEM145::Cm containing the Cm^r cassette in the same orientation as the Cj0145 gene was introduced in *C. jejuni* 81116 by natural transformation.

Construction of PhosR and PhosS overexpression plasmids

To overexpress the complete response regulator PhosR and the cytoplasmic domain of sensor protein PhosS, chromosomal DNA (strain 81116) was amplified using the primer combinations HisCj0890NdeI/Cj0890PstI and HisCj0889NdeI/Cj0889PstI respectively (Table 2). The resulting PCR fragments (662 and 728 bp long respectively) were digested with NdeI and PstI and cloned into the NdeI and PstI sites of pT7.7 to form pT7-890-H6N and pT7-889c-H6N respectively. The nucleotide sequence of the cloned PCR products was verified by sequencing both strands.

Construction of a PhosR complementation plasmid

The Cj1492 promoter, present on a 2549 bp PCR product made with primers Cj1491cR and Cj1491cF (Table 2), was isolated from plasmid pGEM1491 by digesting this plasmid with SalI and MunI. The resulting 1414 bp promoter fragment was ligated into the *Campylobacter* shuttle vector pWM1007 digested with SalI and EcoRI to obtain plasmid pWM1007Pr1492. The intact sensor and regulator genes *phosS* and *phosR* were cloned downstream the Cj1492 promoter by digesting the plasmids pGEM890-889 and pWM1007Pr1492 with SstI and NotI followed by ligation to obtain the complementation plasmid pCP890.

Purification of recombinant proteins

Histidine-tagged PhosS and PhosR proteins were expressed in *E. coli* BL21(DE3) containing plasmid pT7-889-H6N or pT7-890-H6N. Protein expression and purification were performed as described previously (Wösten *et al*., 2004). Protein concentrations were determined using the Bradford method (Bradford, 1976).

Phosphorylation assays

Recombinant PhosSc-H6N (22 pmol) and PhosR-H6N (4.3 pmol) proteins were incubated at room temperature for 15 min, with 10 μCi of [γ-^{32}P]ATP in 40 and 10 μl phosphorylation buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 2 mM $MgCl_2$ and 1 mM DTT) respectively. Phosphorylated PhosSc-H6N was separated from [γ-^{32}P]ATP by ultrafiltration (Microcon YM10; Millipore Corporation, Bedford, MA). Phosphorylation of 4.3 pmol PhosR-H6N was accomplished by adding 22 pmol of autophosphorylated PhosSc-H6N in 80 μl phosphorylation buffer. After 0, 0.1, 0.5, 1, 2, 4, 8 and 16 min, a 10 μl sample was taken and the reaction of this sample was stopped with SDS-loading buffer. Samples were run on 12.5% SDS-polyacrylamide gels. After electrophoresis the gel was dried and autoradiographed.

Growth curve

Overnight cultures of *Campylobacter* grown in defined medium (1.6 mM [P_i]) (Leach *et al*., 1997) were diluted to A_{550} of 0.05 in 10 ml of defined medium containing either 1.6, 0.08 or 0.016 mM [P_i] and incubated at 37°C, 150 rpm under microaerobic conditions (Brazier and Smith, 1989). Throughout the growth cycle, flasks for each time point were removed from their incubation conditions, and 1 ml samples of the cultures were obtained for analysis.

RNA isolation

Precultures of *Campylobacter* grown in HI or defined media (1.6 mM [P_i]) were diluted to A_{550} of 0.05 in 5 ml HI or defined medium (containing either 1.6, 0.4, 0.08 or 0.016 mM [P_i]) respectively, and incubated at 37°C on a gyratory shaker (150 rpm) under microaerobic conditions. Total RNA was extracted from logarithmic phase grown cultures (A_{550}, 0.4–0.7) with the RNA-Bee™ kit (Tel.Test) according to the manufacturer's specifications. Size chromatography of RNA was done with an Agilent 2100 Bioanalyser.

Construction of the C. jejuni *DNA microarray*

DNA fragments of individual ORFs were amplified using ORF-specific primers for those present in strain NCTC 11168 (Sigma Genosys, The Woodlands, TX) and for strain RM1221 using primers from Operon Technologies (Alameda, CA) designed with ArrayDesigner 2.0 (Premier Biosoft, Palo Alto, CA). Additionally, lipooligosaccharide (LOS) genes were amplified from LOS locus classes A, B, C, D, E and F as described previously (Parker *et al*., 2005). Each PCR reac-

Table 2. Primers used in this study.

Primer name	DNA sequence (5′–3′)
Cj0890cF	TTAAATCTATAAGAGCCATT
Cj0890cR	GATGATATTATCAAAGAATT
Cj0890FBamHI	TTAGGATCCAGCAAAAGCTCATTGAAATC
Cj0890RBamHI	CGCGGATCCGCAATTTTAAAACGCCATAA
Cj144F2	GATGTTATCACTCAATCTGAA
trxB	AAAATGTAGTAATGTTTGAAAAAGGTA
Cj1491cR	TGAATTTGATGTGAGTATAGA
Cj1491cF	TAAGGCTAAATCAGTCTTAT
Real-time RT-PCR	
Cj0145Ftaq	AGGGCCTATTGCTTGTGAATTAAC
Cj0145Rtaq	ACCTTCTCCTGGATGTTGTATGC
Cj0429Ftaq	TTAAAGCCGCATTGATAAGATCATA
Cj0429Rtaq	TGATGATGGAGAGCCTAAAGGAA
Cj0613Ftaq	AAACCAATGCTCCATTAAATATCACA
Cj0613Rtaq	GATTTGATTCCAGTTTGTATAAGGATTTG
Cj0614Ftaq	TGGTTTTTGCCGTGATTTTACC
Cj0614Rtaq	TTTCGCCTAAGGCTCTTGAAAG
Cj0615Ftaq	ACTAGCGTTTTGCCTGCTCAA
Cj0615Rtaq	CGCTGCGGTTCTTTCTAAAAAT
Cj0616Ftaq	CATCTCTTATGCACCAAAACTTCATG
Cj0616Rtaq	ACCTCTTCAAAAAGCCCCACTT
Cj0727Ftaq	TGAGTTCAGCTCAAGAAATTGCTAAAT
Cj0727Rtaq	CCAAAAGACGCCCCTACATC
Cj0728Ftaq	GCAGTGTTTTATTTGGAGTTCAAGAGT
Cj0728Rtaq	AGGAGCATCCTTAGGAGGTTTTACT
Cj0729Ftaq	AAACAAGCATTTTTGACCTTTGC
Cj0729Rtaq	TGGCGATGTAAAGAAGGAATAAATT
Cj0730Ftaq	TGTAAAATGTTAGGTGGAGGCAATT
Cj0730Rtaq	TCGCATAAGCTCCAAAAGCA
Cj0731Ftaq	GTGTAAGTGTTTTGCCTGATGATCTT
Cj0731Rtaq	ACTGCCCACACAAACAAGCA
Cj0732Ftaq	GATAGCTTGGGTTTAAAACACGATTT
Cj0732Rtaq	TTTAGCCTCTAAGCCTTCGTTAGAA
Cj0733Ftaq	TTTTTGGGAGTGAGAGAAAGCAT
Cj0733Rtaq	CTTGCCTTCCTTAAAGCTATAAATCC
Cj1012Ftaq	CTGCACATAAAGTTTGGCAAGTTC
Cj1012Rtaq	GCATGTAGCTGAAAAGACTAAAAAATTTG
Cj1148Ftaq	TCGCTTTTTGCGATCTTTTCA
Cj1148Rtaq	GGGCCAAAAATAGCTACGGTTT
Cj1184Ftaq	GCTATGCCAAGAGTAGGTTTAACAGA
Cj1184Rtaq	TCTTTTTTGCTATCACCCACTTGA
ptaFtaq	GCTATGGTTAGTGGAGCATCAACTAC
ptaRtaq	CCTGAAACGGAACTAATACCTTCTTT
adkFtaq	GCTCCAACTAAAACTATCATCATCGA
adkRtaq	TTTGCTCACTTAAAACCTTGTCAAA
Cj0890Ftaq	GAGTTGAAGATTTGCTCGATAATGAG
Cj0890Rtaq	CTGCTTCTTTATATCCTTGCTCTCTAA
Protein expression	
HisCj0890NdeI	CCACATATGCACCATCACCATCAC CATTTGAAATTTAAAATTTTAATTAT
Cj0890PstI	TTTCTGCAGTTAGCATAATTTATAGCCAATA
HisCj0889NdeI	CCACATATGCACCATCACCATCACCAT ACTAAGGATAAAATTCTAAAG
Cj0889PstI	TTTCTGCAGTTATCCTTGACTGAGTTT
Mobility shift assay	
CFTDig	TTTTACAACTTCTTTAGAAAGCAT
pstSF1	GTCACACTTAAAGATAGAATTT
pstSF2	AAACTTGGAAACATTTTGGAAAC
pstSF3	TTGTATTTTTCTTAAATATAATT
Cj144FDig	GATGTTAAGAAGAAGAGGTT
CJ0145R	TTAAAAACAATCTTCTTTCCAT
corAFDig	GAGTTTTGATATAAATATAAAGCAT
Cj0727R	CTTAAGAAAAATTTCTTACTCAT
Primer extension	
Cj0145Prex	TTTGAACTTGCAAAAAAAGCCAC
pstSPrex	CTGCATTTAAAGCACCACAAAGT
Cj0727Prex	ATAAAGCAACAAGACCTAAACT

tion (total reaction volume, 100 μl) consisted of 1× MasterAmp Taq PCR buffer, 1× MasterAmp Taq Enhancer, 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, forward and reverse primers at 0.2 μM each, 0.5 U of MasterAmp Taq DNA polymerase (Epicentre Madison, WI), and approximately 50 ng of genomic DNA from strain NCTC 11168. Thermal cycling was performed using a Tetrad thermal cycler (MJ Research, Waltham, MA) with the following amplification parameters: 30 cycles of 25 s at 94°C, 25 s at 52°C, and 2 min at 72°C and a final extension at 72°C for 5 min. PCR products were analysed by gel electrophoresis in a 1% (wt/vol) agarose gel (containing 0.5 μg of ethidium bromide ml^{-1}) in 1× Tris-acetate-EDTA buffer. DNA bands were examined under UV illumination. We successfully amplified a total of 1530, 227 and 40 PCR products from strain NCTC 11168, RM1221 and the LOS genes respectively, covering 94% and 12% of the predicted ORFs of strains 11168 and RM1221. They were purified on a Qiagen 8000 robot using a Qiaquick 96-well Biorobot kit (Qiagen, Valencia, CA), dried and resuspended to an average concentration of 0.1–0.2 μg μl^{-1} in 20 μl of 50% dimethyl sulphoxide (DMSO) containing 0.3× saline sodium citrate (SSC). All of the PCR probes were then spotted in duplicate on UltraGAPS slides (Corning) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI) producing a final array containing a total of 3594 features.

Microarray hybridization and analysis

For the expression profiling, an indirect comparison of gene expression was performed (Yang and Speed, 2002). In this microarray experimental design, each labelled cDNA was combined with a common reference (labelled genomic DNA), as described in previous studies (Eriksson *et al*., 2003; Lucchini *et al*., 2005). The labelled genomic DNA also served as a quality control for all the spots in the array. Twenty micrograms of total RNA from each *C. jejuni* isolate was labelled during reverse transcription to cDNA with Cy3-dUTP using Stratascript (Stratagene, Palo Alto, CA). Following 16 h labelling, RNA was degraded by the addition of NaOH to 0.3 M and incubation at 70°C for 10 min, followed by neutralization with an equimolar amount of HCl. The labelled cDNA was purified using Qiagen Qiaquick PCR columns according to manufacturer's directions. Genomic DNA from strain 81116 was labelled with Cy5-dUTP. Approximately 2 μg of DNA was mixed with 5 μl 10× NEBlot labelling buffer containing random sequence octamer oligonucleotides (New England Biolabs, Beverly, MA) and water to a final volume of 41 μl. This mixture was heated to 95°C for 10 min and then stored for 5 min on at 4°C. After this incubation, the remainder of the labelling reaction components were added: 5 μl of 10× dNTP labelling mix (1.2 mM each dATP, dGTP, dCTP; 0.5 mM dTTP in 10 mM Tris pH 8.0; 1 mM EDTA), 3 μl of Cy5 dUTP (Amersham Biosciences, Piscataway, NJ) and 1 μl of Klenow fragment. The labelling reactions were incubated overnight at 37°C. Fluorescently labelled DNA was purified using Qiagen Qiaquick PCR columns according to manufacturer's directions.

For each gene expression hybridization, Cy5-labelled reference DNA from strain 81116 was mixed with Cy3-labelled test cDNA in 45 μl of Corning hybridization buffer (Acton, MA) and heated to 95°C for 5 min. Then 15 μl of the hybridization

mixture was put onto the microarray slide and sealed with a coverslip in a Corning hybridization chamber and incubated at 42°C for 18 h. Following hybridization, microarray slides were washed for 2 min in 2× SSC, 0.1% SDS at 42°C to remove the coverslip and then washed twice for 5 min each time in each of the following buffers: (a) 2× SSC, 0.1% SDS at 42°C, (b) 0.2× SSC, and finally (c) 0.01× SSC. Microarray slides were dried by centrifugation at 300 *g* for 15 min before scanning.

DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA) and the data for spot and background intensities were processed using the GenePix 4.0 software. Poor features were excluded from analysis if they contained abnormalities or a reference signal lower than background plus three standard deviations. As described by Eriksson *et al.* (2003), fluorescence ratios were calculated after local background was subtracted from spot signals. To compensate for any effect of the amount of template and uneven Cy-dye incorporation, data normalization was performed as previously described (Anjum *et al.*, 2003; Eriksson *et al.*, 2003).

Normalized data that passed the quality controls were analysed using GENESPRING 7.2 software (Agilent). For the comparison of *C. jejuni* strains 81116 and 81116 *phosR*::Cm gene expression, six hybridization measurements were generated per biological experiment (three technical replicate arrays and two replicate features per array) and the experiment was repeated two times (biological replicates). Significance of the centred data at $P \geq 0.05$ was determined using a parametric-based statistical *t*-test adjusting the individual *P*-value with the Benjamini and Hochberg false discovery rate multiple test correction within the GeneSpring analysis package.

Real-time RT-PCR

Real-time RT-PCR analysis was performed as previously described (Wösten *et al.*, 2004). Primers used in this assay are listed in Table 2. The calculated threshold cycle (Ct) for each gene amplification was normalized to the Ct of the *gyrA* gene amplified from the corresponding sample before calculating fold change using the arithmetic formula ($2^{-\Delta\Delta Ct}$), where $\Delta\Delta Ct = [(C_{t\ target} - C_{t\ gyrA})_{mutant} - (C_{t\ target} - C_{t\ gyrA})_{wild\text{-}type}]$ (Schmittgen, 2001), where target = gene. Each sample was examined in four replicates and was repeated with at least two independent preparations of RNA. Standard deviations were calculated and are displayed as error bars.

Gel mobility shift assays

The promoter region upstream of the genes Cj0145, *pstS* and Cj0727 were amplified by PCR using *C. jejuni* 81116 chromosomal DNA as template. Primers CFTDig, Cj144FDig and corAFDig were digoxigenin labelled (Isogen). The Cj0145 (246 bp), pstS-F1 (232 bp), pstS-F2 (163 bp), pstS-F3 (130 bp) and Cj0727 (163 bp) promoter regions were amplified using primer sets Cj144FDig/CJ0145R, CFTDig/pstSR1, CFTDig/pstSR2, CFTDig/pstSR3 and corAFDig/Cj0727R respectively. Approximately 25 pmol of digoxigenin-labelled pstS-F1, pstS-F2, pstS-F3 DNA fragments and 0, 1, 5, 25 or 50 pmol of PhosR-H6N protein in a 15 μl volume were incubated at room temperature for 15 min. The Cj0145, pstS-F1 and Cj0727 DNA fragments (25 pmol) were incubated with 25 pmol PhosR-H6N and 44 pmol PhosS-H6N with or without 2 mM ATP. For competition gel mobility shift assays 25 pmol PhosR-H6N protein, 25 pmol pstS-F1 DNA and 30 or 180 pmol unlabelled pstS-F1 DNA in a 15 μl volume were incubated at room temperature for 15 min. The binding buffer used for protein–DNA incubations contained 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 50 μg bovine serum albumin, 1.0 μg poly (dI-dC), and 5% glycerol. Samples (20 μl) were run on a 4% non-denaturing Tris-glycine polyacrylamide gel at 4°C. Following electrophoresis, DNA was transferred to nylon membranes (Amersham) and UV cross-linked. Digoxigenin-labelled DNA was detected with anti-digoxigenin antibodies according to the manual of the manufacturer (Roche).

Primer extensions

Analysis of the 5′ ends of the Cj0145, *pstS* and Cj0727 mRNA transcripts was performed by primer extension using [γ^{32}P]ATP labelled primers. A total of 5 pmol of primer Cj0145Prex, pstSPrex or Cj0727Prex (Table 2) was annealed to 20 μg of RNA extracted from *Campylobacter* grown in defined medium with 0.08 mM [P_i]. Synthesis of cDNA was performed using SuperScript™II RnaseH$^-$ reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The extension products were analysed by electrophoresis on a 6% polyacrylamide, 7.5 M urea gel and compared with sequence ladders initiated with primers Cj0145Prex, pstSPrex or Cj0727Prex.

Alkaline phosphatase assay

Optical density of the culture (1 ml) was measured at 600 nm before pelleting. The pellet was resuspended in 0.5 ml of 10 mM Tris/HCl pH 8, 1 mM EDTA, 0.1% SDS and 5 μg lysozyme and incubated for 10 min at room temperature. Alkaline phosphatase activity was assayed by monitoring the release of p-nitrophenol (PNP) from 2 mM p-nitrophenyl phosphate (PNPP) at 37°C and stopped by adding 0.1 M KH_2PO_4. The units of alkaline phosphatase were calculated using the formula $10^3 \times [A_{420} - (1.75 \times A_{550})]/[t \times OD_{600} \times V]$, where A_{420} and A_{550} are the absorbencies of the reaction mix after an incubation time t (min), OD_{600} is the optical density of the culture and V is the volume (ml) of the culture used in the assay (Brickman and Beckwith, 1975)

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